Freshly excavated fossil bones are best for amplification of ancient DNA

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Despite the enormous potential of analyses of ancient DNA for phylogeographic studies of past populations, the impact these analyses, most of which are performed with fossil samples from natural history museum collections, has been limited to some extent by the inefficient recovery of ancient genetic material. Here we show that the standard storage conditions and/or treatments of fossil bones in these collections can be detrimental to DNA survival. Using a quantitative paleogenetic analysis of 247 herbivore fossil bones up to 50,000 years old and originating from 60 different archeological and paleontological contexts, we demonstrate that freshly excavated and nontreated unwashed bones contain six times more DNA and yield twice as many authentic DNA sequences as bones treated with standard procedures. This effect was even more pronounced with bones from one Neolithic site, where only freshly excavated bones yielded results. Finally, we compared the DNA content in the fossil bones of one animal, a ≈3,200-year-old aurochs, excavated in two separate seasons 57 years apart. Whereas the washed museum-stored fossil bones did not permit any DNA amplification, all recently excavated bones yielded authentic aurochs sequences. We established that during the 57 years when the aurochs bones were stored in a collection, at least as much amplifiable DNA was lost as during the previous 3,200 years of burial. This result calls for a revision of the postexcavation treatment of fossil bones to better preserve the genetic heritage of past life forms.

ancient DNA | bone diagenesis | conservation | DNA preservation

Our knowledge of past life forms stems mainly from fossils, the only witnesses of extinct species, the phylogenetic analyses of which were boosted by the discovery that DNA is sometimes preserved in fossils (1). In fact, water-soluble DNA has been shown to persist in fossil bones for up to 130,000 years in temperate regions (2). The analysis of this ancient DNA has the potential to provide answers to archeological, paleontological, and anthropological questions, when the classical approaches of these disciplines cannot do so. During bone fossilization, however, DNA is at least partially degraded and chemically modified. Little is known about the modifications of ancient DNA that lead to its preservation. Thus, ancient DNA analysis constitutes an enormous methodological and conceptual challenge for paleogeneticists. Moreover, despite some spectacular achievements, the failure rate of paleogenetic investigations is high, because DNA preservation is rare, i.e., numerous fossil samples are analyzed, but few sequences are obtained. For example, the success rate of DNA amplification declines with increasing average temperature in the area from which the fossils originate. Whereas 78% (3) and 62% (52–71%; ref. 4) of permafrost samples were reported to be successfully amplified, samples from regions with moderate temperature amplified with a 23–67% success rate (5) and from arid hot climates with a mere 2–4% success rate (5). Temperature has indeed been identified as a key factor in DNA preservation (6) but cannot be the only factor. All factors influencing chemical reactions (e.g., pH, oxidizing reducing potential Eh, irradiation, chemical composition of bone and soil, and hydrology) may play a role in a complex fashion not as yet understood.

It has been shown that bones are locally destroyed by bacteria and fungi (microscopical focal destruction; ref. 7), and that diagenetic alteration is localized, leaving discrete fossilizing regions where fossilization can occur (8). Long-term DNA preservation might be favored within various types of microenvironments with different biological and physicochemical properties, so-called molecular niches (9), which formed in the bones during fossilization. The particular conditions ruling in these niches must slow down DNA degradation processes [e.g., adsorption of DNA to apatite crystallites or clusters of intergrown bone crystals not affected by diagenetic changes, such as those described by Trueman *et al.* (10) and Salamon *et al.* (11); complexation of DNA; low local chemical reactivity, particular local pH and ionic conditions, etc. (9)]. These microenvironmental conditions and the physicochemical conditions prevailing in the macroenvironment in which the fossilization process takes place must be interdependent, suggesting that any drastic modification of the conditions outside the bones can affect the preservation of the DNA within them. Abrupt changes in the macroenvironmental conditions, such as those that occur during the excavation of fossils and their transfer to museums and natural history collections, might constitute such changes in the physicochemical conditions in the microenvironment and might thus have dramatic consequences on DNA preservation. Therefore, we tested systematically the interdependence of DNA preservation and postexcavation treatment by analyzing bones that had experienced different postexcavation preservation conditions. Here we show how detrimental standard postexcavation treatments can be to DNA survival.

Results

To analyze the influence of standard postexcavation treatments on the preservation of DNA within archeological bones, here referred to as "fossil bones," we analyzed in parallel fossil bones from museum collections and freshly excavated bones kept after excavation under conditions that resembled as much as possible those in the sediment. To obtain such freshly excavated bones, two of us (M.P. and E.-M.G.) collaborated closely during the past few years with archeologists and archeozoologists to ensure

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Abbreviation: UQPCR, uracil-DNA-glycosylase-coupled quantitative real-time PCR.

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any fossils destined for ancient DNA analysis were handled in a specific way to prevent the growth of microorganisms that would destroy preserved biomolecules and/or their chemical degradation by oxidation and hydrolytic processes. To prevent dissolution and degradation of endogenous DNA and contamination by exogenous DNA, we avoided treatments normally used by archeologists, such as washing, brushing, and treatment with consolidants and other chemicals. The fossil bones were excavated, handled (including paleontological analysis), and stored in an aseptic manner, thus reducing the risk of contamination with human, food- and pet-derived, and environmental DNA. We hereafter call these fossil bones "fresh," as opposed to "old" fossil bones, which had been washed, handled, and stored for many years in collections in a dry state at room temperature.

We obtained evidence for the detrimental effect of standard excavation treatments with fossil bones when we compared the PCR amplification success rate from a large-scale study of 247 herbivore bones, from 600 to 50,000 years old, from various depositional contexts of 60 different archaeological and paleontological contexts in Northern and Southern Europe, the Near and Middle East, and the Arabian peninsula [supporting information (SI) Table 2 and M.P., R.S., V.B.C., and E.-M.G., unpublished work]. We amplified the hypervariable region of the mitochondrial DNA using uracil-DNA-glycosylase-coupled quantitative real-time PCR [UQPCR (12, 13)] and evaluated the PCR and cloning products obtained from at least two independent fossil bone extractions. We found that 46% of fresh fossils yielded authenticated amplification products, whereas 18% of old fossils yielded these products (see SI Table 2). The difference was statistically significant [P (χ^2) = 0.001]. Furthermore, using UQPCR, we could measure the quantity of DNA molecules. We could also estimate that the number of maximal 153-bp-long molecules that could be amplified was on average approximately six times higher in the fresh than in the old fossils (see SI Table 2). Again, despite the wide variations in DNA quantity from various bone samples, this difference was statistically significant [P (t test) = 0.043]. Interestingly, although it was possible to amplify the larger DNA fragments (201 bp) from 15% of the fresh bones, this was the case in only 4% of the old bones. To conclude, we have obtained clear evidence that DNA preservation is better in freshly excavated untreated bones, and that postexcavation treatments and/or storage conditions negatively influence DNA preservation. Because taphonomic conditions drastically influence DNA preservation, one needs to analyze bones whose fossilization has occurred under comparable taphonomic conditions to clearly establish postexcavation conditions as the cause of DNA degradation.

We therefore studied more comparable situations, i.e., bones collected under various conditions from the same preservation site (Telleilat-Mezraa, a Neolithic site in Turkey). We compared the level of DNA amplification of the hypervariable region of mitochondrial DNA from two different fossil bone groups. The old ones had been excavated several years before and had been subsequently brushed with water, dried, and stored under light-exclusion conditions in collections at room temperature; the fresh ones originated from the same archeological site but had been recently excavated according to strict protocols designed to optimize recovery of biomolecular evidence. Here, the difference in the success rate was striking; it was possible to amplify DNA from five of eight fresh fossil bones (with quantities of 39,965–1,634 molecules per gram of bone) as opposed to 0 of 11 old fossil bones. Thus, when the analyzed bones came from the same preservation context, suitable postexcavation conditions were important for DNA preservation. The detrimental effect of postexcavation conditions on DNA preservation was more pronounced than when multiple bones from multiple sites were analyzed. Yet, these bones belonged to different individuals that had died in different ways and therefore had not experienced an identical fossilization history, which is presumably unique for each fossil assemblage from a single organism.

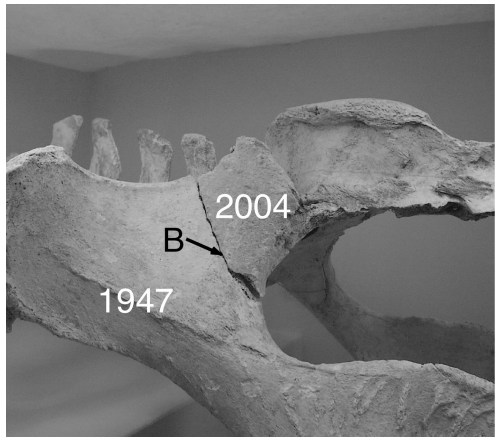

Fig. 1. The hip of the fossil *B. primigenius* specimen from Pontvallain showing a bone fragment excavated in 1947 and one excavated in 2004, perfectly fitting at the line of breakage (arrow).

We obtained final and conclusive evidence from the analysis of exceptional fossil material that shared the same diagenetic history but had experienced different postexcavation histories. We analyzed ribs from an individual aurochs excavated in two different campaigns, the first in 1947 (14) and the second in 2004. The fossil bones excavated in 1947 from a deep karstic crevice in Pontvallain (Pays de la Loire, France) had been stored in the collections of the natural history museum of Le Mans (Musée Vert). In 2004, the crevice was reinvestigated, and 120 additional bones and teeth were recovered. Direct skin-to-fossil contact was carefully avoided, and the specimens were immediately stored at −20°C. The assemblage of the well preserved bone fragments belonged to a single adult individual. One of the 2004 fossil bone pieces perfectly refitted a 1947 fossil bone fragment of the hip of the aurochs (Fig. 1). The genetic analysis of both types of fossils was carried out on the same skeletal elements (ribs) to minimize preservation differences because of anatomical and local geochemical differences. No PCR amplification of the bovine mitochondrial D loop was obtained from the 10 samples from the shafts of two ribs excavated in 1947, despite numerous attempts and a negligible inhibitory effect of the extracts. In contrast, the nine samples from the shafts of three ribs excavated in 2004, with a success rate of 100%, yielded a 153- and a 201-bp amplification fragment of the bovine mitochondrial D loop using UQPCR. The quantity of DNA amplified from the various extracts varied from 1 to 511 molecules per amplification reaction (average of 61 molecules per reaction, ±55.5).

From the fossil bones of the aurochs in Pontvallain, we obtained an aurochs sequence identical to two of the sequences obtained from two older British aurochs fossils [7,500 and 11,900 yr old (15); see Table 1]. The phylogenetic position of the retrieved sequence proves its authenticity. Thus, our study demonstrates that ancient DNA preserved for thousands of years in fossil bones can be degraded relatively quickly when the bones are removed from the preserving conditions of their original setting. This degradation is not the consequence of any differences in the fossilization process but is clearly due to changes in the macroenvironment and/or standard handling and storage procedures in natural history collections. We then analyzed whether the different postexcavation treatments of these bone samples left any hallmarks of distinctive morphological changes using light microscopy and environmental scanning electron microscopy with backscattered electron detectors (ESEM), which makes its possible to characterize and quantify the histo-

Table 1. Bovine DNA sequences of the mitochondrial D loop

Nucleotide position	49	50	51	55	57	58	74	82	84	85	92	102	109	110	113	116	117	119	121	122	130	135	137	138	141	143	147	185	196	197	201	229	231	247	248	255	264	300	301	302	3
European	C	C	T	T	G	C	T	G	C	T	A	G	T	C	T	T	G	T	G	T	T	T	T	T	T	A	T	G	G	G	*	A	C	C	C	T	G	A	C	G	
African	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	
Indian	T	.	.	.	A	T	.	A	.	.	.	A	C	.	C	C	A	C	A	C	.	C	C	C	.	*	C	.	A	.	A	G	.	T	T	.	.	G	T	.	
D740	T	.	C	C	.	T	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	A	.	.	.	
D812	T	.	C	.	.	T	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	A	.	.	.	
CHWF	T	T	C	.	.	T	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	A	.	.	.	
NORF	T	.	C	.	.	T	C	.	.	C	.	.	C	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	A	.	.	.	
CPC98	T	.	C	.	.	T	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	T	.	.	C	A	.	T	.	
TP65	T	.	C	.	.	T	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	A	.	.	.	
PVL04	T	.	C	.	.	T	C	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	C	A	.	.	.	

European (23), African (24), and Indian (24) consensus sequences; British Pleistocene aurochs sequences (D740, D812, CHWF, NORF, CPC98, and TP65) (15); and PVL04, sequence obtained from the aurochs of Pontvallain (this study).

logical changes that occur during bone diagenesis (16, 17). Under the light microscope, the fossil bones showed no signs of human or carnivore activity. ESEM analysis of bone sections revealed the shape and distribution of internal porosity were very similar in all analyzed specimens, with no apparent difference in texture between the fossil bones recovered in 1947 and in 2004 (see Fig. 2). Histological traits were identical in all samples, and they all showed extensive bacterial attack. Neither type of fossil bone showed any cracking or exfoliation on the surface or at the sections. The absence of any such alterations indicates there were no differences in weathering stages, humidity/dehydration, dryness, compacting, or deformation between the two types of fossil bones (see Fig. 2 *B* and *D*). No traits of specific preparation and/or conservation treatment [e.g., chemical solutions to clean the fossils or act as preservatives/consolidants (18)] were detectable. The elemental composition, as revealed by wavelength- and energy-dispersive x-ray spectrometrical analysis, provided homogeneous spectra of the calcium and phosphate composition in both the 1947 and 2004 fossil bones. In summary, there is no microscopic indication that samples obtained from excavations in 1947 and 2004 underwent different diagenetic processes that may suggest differences in their taphonomic history. There are also no microscopic differences between the two types of fossil bones that could be attributed to particular treatments or conditions during storage of the fossil bones after excavation from the site of Pontvallain in 1947.

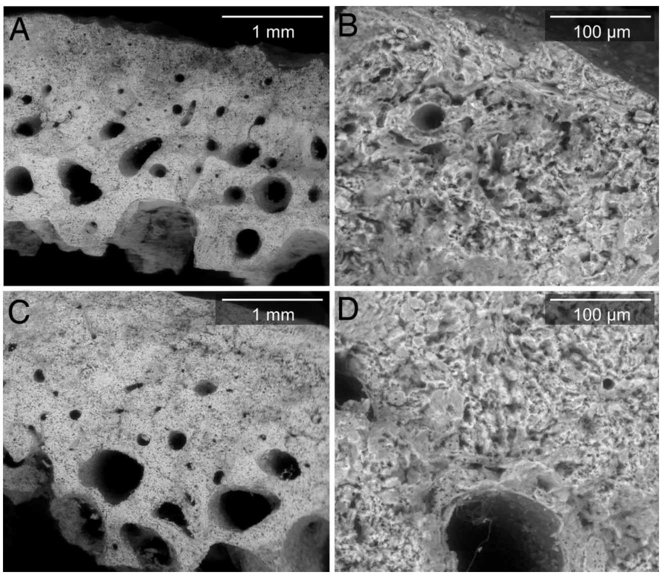

Fig. 2. Scanning electron microphotographs from cross-sections of two different fossil bone fragments from the aurochs ribs recovered from Pontvallain in 1947 (*A* and *B*) and in 2004 (*C* and *D*) (*A* and *C*, ×40; *B* and *D*, ×400).

Discussion

DNA preservation occurring in postmortem bone must be influenced by many different parameters. Approximately three different preservation phases can be distinguished. During the first diagenetic phase, the bone undergoes bacterial putrefaction. This is a rapid, complex, and multicomponent process. We estimate that the putrefaction phase can cause a 15-fold decrease in the quantity of amplified DNA, because we were able to measure 1.5×10^6 molecules per gram of fresh bone and only 1×10^5 molecules per gram of bones from a recent (≈20-year-old) bone that had completed the putrefaction phase. This bone was a naturally and manually defleshed humerus from the carcass of a Batina zebu.

If, at the end of the putrefaction phase, the conditions are favorable for long-term preservation of organic matter, the bone will enter diagenetic phase 2, and DNA degradation will continue mainly on a chemical basis. One of the major DNA degradation pathways is depurination (19). This degradation reaction probably follows a first-order kinetics model in which $[A]/[A_0] = e^{-kt}$, where $[A]/[A_0]$ is the fraction of remaining material, k is the degradation rate, and t is the time. We estimated the fraction of remaining material by comparing the quantity of PCR-amplifiable DNA contained in both the recent postputrefaction bones and the fossil bones from the 3,200-year-old Pontvallain aurochs. From the freshly excavated fossil bones of the latter, we amplified 100-fold less than from the postputrefaction bones, i.e., an average of 2,547 ± 5,835 mitochondrial DNA molecules per gram of bone. These quantities indicate the degradation rate of DNA in the aurochs bones during burial was ≈90% per 2,000 years.

That we did not obtain any PCR product from other ribs belonging to the same individual, unearthed 57 years ago and subsequently stored in the natural history collection, indicates that at least 99% of the DNA was degraded during this period, which corresponds to diagenetic phase 3. This means that the degradation rate was at least 70 times faster during the 57 years after excavation than during the ≈3,200-year-long burial phase. The corresponding degradation rate of 90% per 30 years is comparable to that described for recent fox teeth, which had been autoclaved after the animal's death and conserved during the first 30 years in a museum (20), and for which a DNA degradation rate of 90% per 15 years can be estimated. The

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slower DNA degradation rate of 90% per 2,000 years estimated for the buried aurochs bones from Pontvallain compares well with the depurination rate of DNA in solution obtained by Lindahl and Nyberg (19) applied to a 150-nt-long molecule at a temperature of 5–10°C (temperature in the burial environment, measured during excavation) and at neutral pH (see calculations in *Materials and Methods*). According to Lindahl and Nyberg's measurements (19), however, an increase of 15°C in the average temperature (which we assume to be ≈20–25°C in a museum) would be enough to accelerate the degradation rate 16 times. Furthermore, additional modifications of the pH and the ionic strength could further raise the DNA degradation rate to 70-fold. A decrease in the pH from 7.4 to 6.4 increases the depurination rate 3.3 times (19). Washing of the aurochs bones (buried in sediment of pH 7.5) with tap water, which today typically has a pH of ≈5.5, is likely to decrease the pH to an unknown extent, because the mineral part of the fossil bone has probably retained some of the buffering capacity of the bioapatite of fresh bone. A decrease in salt-content as a consequence of washing could also be responsible for an increase in the DNA degradation rate, because depurination is 7-fold faster when the concentration of NaCl is decreased from 0.1 to 0 M (19). Finally, washing of the fossil bone could have dissolved the most soluble parts of the DNA. Thus, the rapid degradation of DNA observed in the aurochs bones after their excavation is compatible with the effects of the standard washing procedures for fossil bones routinely used by archeologists and paleontologists, combined with an elevation in temperature in the storage room.

Bone is a very heterogeneous tissue with unevenly distributed biological and physicochemical properties, and bacterial attack will not be homogeneous. Furthermore, local differences in physicochemical properties should also influence long-term DNA preservation and, indeed, we and others (21) have observed local heterogeneity in DNA preservation within a bone. We were able to ascertain that the differences in DNA preservation observed with the Pontvallain aurochs bones were not due to such local heterogeneity in DNA preservation, because the preservation of DNA proved to be similar within each series of bone samples from each category (three fresh and two old ribs) and was different between categories.

Whatever the exact causes, our results show very clearly how detrimental standard treatments are to the preservation of DNA in fossil bones. When classifying the 247 fossil herbivore bones analyzed (all of which experienced differences in diagenetic phases 1 and 2) into two categories according to postexcavation treatment (standard vs. special), the PCR success rate for the bones excavated under special conditions was twice that of those treated normally. Moreover, these standard excavation and storage conditions reduce the quantity of DNA by a factor of about six. These conditions are particularly detrimental when the quantity of DNA contained in the bones is already low, as in the case of most fossil bones. In fact, treatments that result in a 6-fold reduction in DNA quantity would cause the failure of PCR amplification in 3 of the 15 PCR-positive fossil bones excavated under special conditions (see SI Table 2). This reduction would be sufficient to reduce the percentage of PCR-positive bones among the fresh bones to a level not significantly different from that observed in the bone sample excavated under standard conditions. Thus, the average decrease in the quantity of amplifiable DNA could be sufficient to explain the difference in the PCR success rate.

The effect of postexcavation treatment was found to be even more pronounced in bones preserved in the same burial site (Telleilat-Mezraa). These bones had undergone a different diagenetic phase 1 and a similar diagenetic phase 2 and had then experienced a different postexcavation phase 3. Here we observed a striking difference in the PCR success rate among the fresh recently excavated fossil bones (63%) compared with those excavated under standard conditions (0%).

Finally, when we compared the fossil bones from the same animal and the same burial site (the aurochs from Pontvallain), which had experienced differences in diagenetic phase 3 only, we also obtained a spectacular result with a 0% amplification success rate in the case of the old and a 100% amplification success rate in the case of the fresh fossil bones. This result is ultimate proof of the detrimental effect of standard postexcavation treatments of fossil bones on the survival of amplifiable DNA.

In conclusion, even if amplification results from collection fossil bones can be obtained, fewer fossil bones will yield PCR results, and less DNA will be retrieved. This effect will be more pronounced the less DNA is preserved in the fossil, thus leading to a north–south gradient of suitability of fossils for paleogenetic studies, for which fossils from permafrost areas and cold caves are more suitable than those from hot and dry climate zones. Our finding has major implications for paleogenetic studies, which are a key to the study of extinct species and populations and can reveal the mechanisms leading to extinction. We propose that recently excavated and untreated fossil material should be preferred to fossil material that has been washed, treated with chemicals, and stored for a long time in regular museum collections. Furthermore, excavation, preparation, conservation protocols, and storage conditions for fossil bones in collections should be revised if genetic information is to be preserved and retrieved. If, at a given archeological or paleontological site, paleogenetic results are to retain their potential to answer archeological, paleontological, and biological questions, a selected number of the fossils should not be subjected to any treatment but instead stored in the cold, at least in a cold room, preferably in a freezer, and ideally in a cryobank, in small aliquots to avoid repeated freezing and thawing cycles of the same sample. This approach calls for close collaboration among paleogeneticists, paleontologists, archeologists, conservation managers, and curators.

Materials and Methods

Samples, Fossil Excavation, and Storage Procedure. The aurochs bone samples (here called fossils) used for this study originate from a paleontological site in France (Pontvallain, La Sarthe). Moreover, the results of a study of 247 bovine and equine bones ≈600 to ≈50,000 years old, originating from France, Germany, Switzerland, Spain, Georgia, Armenia, Turkey, Syria, United Arab Emirates, and Bahrein are discussed. The specimens examined include bones excavated using standard archeological and paleontological field procedures and those excavated under strict protocols designed for bones destined for DNA analysis. SI Table 2 summarizes the provenance, contexts, and treatment of the specimens.

We analyzed samples of five rib shafts (diaphyses) from the skeleton of a ≈3,200-year-old aurochs buried in a crevice in Pontvallain (La Sarthe, France), two excavated in 1947 and three in 2004. The climatic conditions in this geographic region correspond to a moderate oceanic climate type with an annual rainfall of 678 mm (45–70 mm per month), and temperatures range from 4°C to 19°C throughout the year. The temperature in the crevice was between 5°C and 10°C. The fossils excavated in 1947 were kept in a dry state in cardboard boxes and drawers in Musée Vert in Le Mans. Environmental records kept in the museum since 1995 show that the mean temperature during the year varies between 15°C and 25°C and the relative humidity, between 40% and 60%. The storage conditions of the bone collection before 1995 were not controlled but were rather those of basic uninsulated stores typical for fossil bone collections in the past, where temperature possibly fluctuated between 0°C and 40°C and relative humidity, between 20% and 90%. The fossil bones newly excavated in 2004 were subjected to

strict protocols; i.e., specimens were handled with gloves; were not washed, brushed, or treated with consolidants or other chemicals; and were immediately frozen at −20°C, surrounded by their sediment. The circumstances of recovery and subsequent handling of this skeleton are discussed in detail in *Results* and *Discussion*.

Eight of the fossil bones from the Turkish Neolithic site of Telleilat-Mezraa were excavated in 2002 by using this strict protocol. Eleven old fossil bones from this site had been excavated between 1992 and 2003. These old bones were excavated according to standard archeological field procedures and had been brushed in water, sun-dried, and stored in cardboard boxes, first in Turkey (with fluctuations in temperature from 0°C to 30°C) and then in Southern Germany. The climate in the area of Telleilat-Mezraa is characterized by an average temperature of 16.7°C (average high temperature of 23.9°C and average low temperature of 9.3°C), annual precipitation of 21.3 mm, and mean humidity of 56.2% (weather station of Bireçik, Turkey).

Samples from the diaphysis of the unburied humerus from a Batina zebu that had died naturally and that had undergone putrefaction were collected from the surface in Muscat in North Oman in 1983. This specimen was already naturally putrefied, almost totally defleshed (remnants of flesh were removed manually), and stored in the bone collection of the University of Tübingen, Tübingen, Germany.

A fresh cow bone was obtained from a butcher and frozen at −20°C until analysis.

Dating of the Aurochs Bones from Pontvallain. ^{14}C dating of one bone sample gave an uncalibrated radiocarbon age of 3,204 ± 56 years. Radiocarbon dating was performed on extracted pure collagen (C/N ratio of 2.8) by the Physical Institute of the University of Erlangen–Nürnberg (Erlangen, Germany).

Taphonomic Analysis of the Aurochs Bones from Pontvallain. The aurochs bones from Pontvallain were analyzed to identify any possible preburial treatment such as boiling, burning, or digestion and any postexcavation (conservation) treatment with glue, resins, varnish, consolidants, or washing with alkaline, acidic, or peroxide solutions, formol, alcohol, or acetone, as described (18). We carried out surface analysis of the fossil bones, avoiding any such treatment and using both a binocular microscope [×0.7 to ×80 Leica (Eindhoven, The Netherlands), MZ 7.5] and environmental scanning electron microscopy (QUANTA 200 Environmental Scanning Electron Microscope, Philipps, Amsterdam, the Netherlands). The analysis of the fossils was thus possible without additional preparation, as described (18). The elemental composition of the samples and identification of inclusions and mineralization were analyzed by using wavelength- and energy-dispersive x-ray spectrometry.

DNA Extraction. All pre-PCR work was carried out in a physically isolated work area in a part of the building (basement) where no other DNA work was done. The cleaning and powdering steps were performed in an area dedicated to work on fossil bones separate from the laboratory where DNA was extracted. For the fossil aurochs bones from Pontvallain excavated in 1947 and 2004, the middle parts of the rib shafts were analyzed. The fossil bones were cut and the surface removed in a UV-irradiated glove box. They were then ground to a fine powder in a freezer mill (Freezer Mill 6750, Spex Certiprep, Metuchen, NJ). Further processing of the bone powder was performed in a laboratory dedicated to ancient DNA work (fossil laboratory), as described (refs. 12 and 13; see also *SI Text*). Blank extractions were carried out for each extraction series.

DNA Amplification. PCR amplification and experiments with modern as well as amplified and cloned DNA were carried out in three different laboratories not in the same part of the building as the fossil laboratory. To reduce the number of potential sources of error-prone sequences, we used the quantitative real-time PCR approach, UQPCR (13). Thus, for each fossil extract and PCR amplification, we (*i*) quantified the target molecules present in a given fossil extract, (*ii*) diluted the fossil extract to abolish its inhibitory power as evaluated by the amplification of an external reference DNA, and (*iii*) destroyed with uracil-*N*-glycosylase (UNG) potential previous PCR and cloning products, thereby avoiding carryover contamination. All PCR amplifications were performed in the Light Cycler (Roche Applied Science, Indianapolis, IN) in individual glass capillaries using UQPCR, as described (ref. 13; see also *SI Text*). Reamplifications were never performed. Quantification of the target molecules in the extracts was performed as described (13).

A total of 29 PCRs (9 with primer pair BB1/2 and 20 with primer pair BB3/4) were performed on 10 independent extracts from samples of the shafts of two different ribs of the aurochs excavated in 1947. Nine extracts from nine samples of the shafts of three different ribs of the aurochs excavated in 2004 were amplified in 48 PCRs with primer pair BB3/4 and 12 PCR amplifications with primer pair BB1/2.

Authentication of the Ancient DNA Sequence of the Aurochs of Pontvallain. (*i*) The nine DNA extractions from the rib samples of the aurochs from Pontvallain excavated in 2004 were amplified in 60 reactions by using UQPCR (13) with two primer pairs from the hypervariable control region of bovine mitochondrial DNA and yielded a PCR product. Blank extractions, performed with each fossil extraction, yielded no amplification products. Negative controls, performed for each PCR amplification, were always negative. (*ii*) The initial quantity of target molecules and quality of the results were assessed as described (12, 13) and were on average 761 ± 715 molecules per gram of fossil powder from rib 1, 2,675 ± 3,164 molecules per gram of fossil powder from rib 2, and 4,926 ± 9,744 molecules per gram of fossil powder from rib 3 of the aurochs from Pontvallain (based on the assumption that one cell contains 1,000 mitochondrial genomes). Sequences of amplification products obtained from a small number of starting molecules were compared with those starting from 100 authenticated mitochondrial molecules and were found to be identical. (*iii*) For each fossil extract, the inhibitory effect was assessed on the basis of the decrease in the PCR efficiency and the amplification delay of modern genomic bovine DNA. The inhibiting extracts were diluted until the inhibition of the amplification reaction was abolished, i.e., in general, 1:2. (*iv*) Before each PCR amplification, the products of previous PCR amplifications as well as cloned DNA were destroyed, and deaminated cytosines were eliminated with UNG (13). (*v*) Sequencing was carried out directly on the PCR product itself and on several clones of the PCR products; 29 PCR products (20 BB3/4 and 9 BB1/2) and 34 clones (25 BB3/4 and 9 BB1/2) were sequenced. Identical *Bos primigenius* sequences were obtained, except for one clone from the BB3/4 products, which showed a mutation (A→G in position 16043). (*vi*) PCR amplifications were repeated in a different laboratory (Genoscope, Evry, France), and identical sequences were obtained. (*vii*) A deer bone from the same excavation site yielded a *Cervus elaphus* sequence that showed two mutations when compared with the *C. elaphus* sequence that had been published (22). (*viii*) No contamination by cloned products was observed, as assayed for by amplifying the PCR-positive extracts with primers hybridizing to the cloning vector on either side of the cloned fragment (13).

Calculation of the Depurination Rate of DNA. We used the classical Arrhenius formula to determine the reaction rate (k): $k = A\,e^{-Ea/RT}$, where R is the universal gas constant, T is the absolute temperature, and A is the Arrhenius constant, which relates to the geometric requirements of the reaction and must be deter-

mined experimentally. The activation energy *Ea* for the depurination reaction is 130 kJ/mol (19), and *A* can be estimated from data in ref. 19 to be $2.46 \times 10^{11}\cdot s^{-1}$ at pH 7.4. The depurination rate *k* at 37°C, pH 7.4, is $3 \times 10^{-11}\cdot s^{-1}$ (20) and therefore can be calculated to be $4 \times 10^{-12}\cdot s^{-1}$ at 25°C, $2.5 \times 10^{-13}\cdot s^{-1}$ at 10°C, and $9 \times 10^{-14}\cdot s^{-1}$ at 5°C. Assuming one depurination event in a DNA target molecule within the region to be amplified is sufficient to prevent amplification, the inactivation rate of one strand of a 150-nt-long DNA fragment at 10°C and pH 7.4 would therefore be $1.2 \times 10^{-3}\cdot yr^{-1}$. Using these parameters and the first-order decay formula $[A]/[A_0] = e^{-kt}$ (see *Results* and *Discussion*), it can be estimated that 90% of the DNA molecules would be inactivated in 1,900 years.

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